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Monitoring of hepatitis B virus surface antigen escape mutations and concomitantly nucleos(t)ide analog resistance mutations in Turkish patients with chronic hepatitis B

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SUMMARY

Background: The hepatitis B virus (HBV) polymerase gene completely overlaps with the envelope gene. In the present study we aimed to monitor the prevalence and pattern of the typical mutations for hepatitis B surface antigen (HBsAg) escape, and concomitantly nucleos(t)ide analog (NUC) resistance mutations, in Turkish patients undergoing different antiviral therapies and in treatment-naïve patients with chronic hepatitis B (CHB).**Methods:** The investigation was undertaken between March 2007 and August 2009 and involved a total of 142 patients under NUC therapy (88 males; mean age 42 years (range 13–68); hepatitis B e antigen (HBeAg) negativity in 94 patients; HBV DNA median log 4.3 log₁₀ IU/ml (range 2.0–>6.0); alanine aminotransferase (ALT) median level 76.1 IU/ml (range 12–1082)) and 185 treatment-naïve CHB patients (120 males; mean age 39 years (range 1–76 years); HBeAg negativity in 132 patients; HBV DNA median log 3.5 log₁₀ IU/ml (range 2.0–6.0); ALT median level 60.7 IU/l (range 8–874)).**Results:** The overall prevalence of typical HBsAg escape mutations found in the CHB patients was 8.3% (27/327). In the NUC therapy group the prevalence was 8.5% (12/142), with the following patterns: sY100C + sI110V, sL109I, sP120T, sP127T, sG130R + sG145X, sS132A + sY134N, sY134N + sG145R, sC137G, sD144E, sG145R. In the treatment-naïve group the prevalence was 8.1% (15/185), with the following patterns: sL109I, sI110V, sS117INST, sP120T, sP127T, sM133I, sC137L + sG145R, sS143L. However, NUC resistance mutations were found in 7.7% (11/142) of the patients on NUC therapy and 3.8% (7/185) of the treatment-naïve group patients. Interestingly, the treatment-naïve patients had preexisting drug resistance mutations related to lamivudine (rtL180M + rtM204I), adefovir (rtA181V, rtQ215S, rtI233V), entecavir (intermediate susceptibility with rtL180M + rtM204IHBV variant), telbivudine (rtL180M + rtM204I), and tenofovir (rtA194T).**Conclusions:** The findings of this study show preexisting typical HBsAg escape and NUC resistance mutations are possible. The genetic arrangement of the HBV genome with polymerase and surface genes overlapping has substantial public health and diagnostic implications and relevance.

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Introduction

The hepatitis B virus (HBV) genome is one of the smallest viral genomes (approximately 3200 base pairs) and encodes only one viral enzyme, namely the HBV reverse transcriptase (RT). Like the HIV RT, the HBV RT is an error-prone enzyme lacking proof-reading activity. In combination with a high virus production, this results in HBV quasi-species.¹ The use of antiviral agents

licensed for the treatment of chronic HBV infection, namely nucleos(t)ide analogs (NUCs), can lead to the development of resistance. Additional, compensatory mutations then accumulate that help to restore full replicative capacity. It is important to keep in mind that the HBV polymerase gene completely overlaps the envelope gene.² Mutations in and around the major neutralization domain of HBV, known as the 'a' determinant, may (1) result in HBV reactivation by escape mutants in previously antibody to hepatitis B surface antigen (anti-HBs) immune persons, (2) cause diagnostic problems, and (3) result in failure of infection prevention with vaccination or hepatitis B immunoglobulin (HBIG).^{3–5}

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The main identified hepatitis B surface antigen (HBsAg) escape mutations are sP120T, sM133I, sS143L, sD144A/E, sG145R, sE164D, sW172*, and sW182*, which is the most common pattern.^{4,6,7} Vaccine/HBIs escape mutations sG145R and sP120T in combination with lamivudine (LAM)-associated resistance mutations are often seen in HBV-mono-infected patients following LAM or HBIs treatment.⁸ The mutation selected during NUC resistance can cause concomitant changes in the overlapping reading frame with the major resistance mutations for LAM, adefovir (ADV), and entecavir (ETV), in particular altering the C-terminal region of HBsAg.⁹ Studies have shown that LAM-resistant HBV (rtV173L + rtL180M + rtM204V) has significantly reduced anti-HBs binding due to changes in the HBsAg.^{2,10} However, the point mutation that causes the rtA181T change in the polymerase also encodes a stop codon (sW172*) in surface proteins.¹¹ A recent report has provided evidence for the involvement of HBV encoding the rtA181T/sW172* mutation in the pathogenesis of, and progression to, hepatocellular carcinoma (HCC).¹²

Turkey is an intermediate endemicity area (2–7%) with approximately 6500 individuals newly infected with HBV each year.^{13–15} A recent study in Turkey found an overall prevalence of 4.19% for HBsAg at 22 Red Crescent Centers between 1989 and 2004,¹³ but the prevalence rates seen in southeastern Turkey are highest (9–11%).¹⁶ Moreover, many studies have indicated that Turkish patients with chronic hepatitis B (CHB) show very little genotypic heterogeneity.^{17,18} In fact, according to a complete HBV genome sequence study, most of the HBsAg carriers in Turkey have a very similar S gene sequence (subtype ayw2).¹⁴ However, little is known about HBsAg escape mutations. An HBV vaccine escape mutation (sT143stop codon) was recently identified for the first time in Turkey in a child with CHB.¹⁹ A study sequencing the amplified surface gene region has suggested sM125T and sT127P mutations as HBsAg escape mutations in Turkish patients with CHB and their family members.¹⁵ Another recently published paper described a diagnostic HBsAg escape mutation (sS143L) causing chronic HBV infection in a previously vaccinated treatment-naïve Turkish patient.²⁰

The aim of the present study was to monitor the prevalence and pattern of the typical HBsAg escape mutations, and concomitantly drug resistance mutations, in NUC therapy patients and treatment-naïve patients with CHB in Turkey.

Materials and methods

Patients

The study was carried out between March 2007 and August 2009 at Kocaeli University Hospital and involved those patients who were starting NUC therapy and those who were already under treatment during this time period. All HBV DNA-positive samples detected by real-time PCR were included. In three patients with undetectable serum HBV DNA levels in the real-time PCR, detection was by an in-house HBV PCR. A total of 327 patients were included in the study; 142 patients were already undergoing NUC therapy and 185 were treatment-naïve CHB patients. The patients in the NUC therapy group were being treated with oral LAM (Zeffix® 100 mg/day, GlaxoSmithKline, Middlesex, UK), ADV (Hepsera® 10 mg/day, Gilead Sciences, Inc., Foster City, USA), ETV (Baraclude® 0.5 mg/day and 1 mg/day, Bristol-Myers Squibb Co., Princeton, USA), or tenofovir (TDF; Viread® 245 mg/day, Gilead Sciences, Inc., Foster City, USA) as monotherapy or combination therapy. All of the patients were categorized as chronic HBV carriers according to the European Association for the Study of the Liver (EASL) clinical practice guidelines.²¹ Liver damage was determined according to Knodell's classification.²² 'Add-on' and 'switch' NUC therapy strategies were used in the gastroenterology

and infectious diseases departments. In those patients who were receiving NUC therapy, the timing of the serum sample collection was according to virological and/or biochemical breakthrough. Blood samples were immediately separated by centrifugation, and the separated sera aliquoted and kept at –20 °C until testing.

Serological markers of HBV (HBsAg, anti-HBs, antibodies to hepatitis B core antigen (anti-HBc), hepatitis B e antigen (HBeAg), and antibody to hepatitis B e antigen (anti-HBe)) were tested using commercially available microparticle enzyme immunoassay kits (AxSYM, Abbott Laboratories, IL, USA and Elecsys, Roche Diagnostics, Mannheim, Germany). However, serological data showed that all patients were HBsAg-positive and that hepatitis C virus (HCV; AxSYM, Abbott Laboratories, IL, USA and Elecsys, Roche Diagnostics, Mannheim, Germany), hepatitis D virus (HDV; Dia.Pro Diagnostic Bioprobes, Milan, Italy), and HIV (AxSYM, Abbott Laboratories, IL, USA and Elecsys, Roche Diagnostics, Mannheim, Germany) markers were negative. None of the patients had received the HBV vaccination before their diagnosis of chronic HBV carrier. Clinical features of the study patients according to the EASL clinical practice guidelines are shown in Table 1.

DNA isolation and HBV DNA assay

HBV DNA was isolated from serum samples by a biorobot workstation using magnetic particle technology (NucliSENS-easy-MAG, bioMérieux, Boxtel, the Netherlands). HBV DNA was detected and quantified by a commercial real-time PCR assay (Iontek Biotechnology Inc., Istanbul, Turkey; iCycler iQ5, BioRad Laboratories Inc., CA, USA). An HBV nested PCR was also used, as previously reported by Karatayli et al.²³

Sequencing of the HBV polymerase gene region

Briefly, a pair of primers was designed (forward: 5'-TCGTGGTGGACTTCTCTCAATT-3' and reverse: 5'-CGTTGACAGACTTCCAATCAAT-3') for the HBV polymerase gene region. The PCR conditions for the polymerase gene segment were as follows: 95 °C for 15 min, and 45 cycles consisting of 95 °C for 45 s, 56 °C for 45 s, and 72 °C for 45 s. The final concentration of primer pairs was 0.3 µM. The size of the amplicon in HBV was around 742 bp, and included all the known NUC resistance mutations in HBV. All PCR products were purified using the High Pure PCR Products Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany) and directly sequenced with the ABI PRISM 310 Genetic Analyzer using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech Inc., Piscataway, USA). For the cycle sequencing the following thermal protocol was used: 35 cycles consisting of 95 °C for 20 s, 50 °C for 25 s, and finally 60 °C for 2 min. The reverse primer was used as the sequencing primer at a final concentration of 0.5 µM. The electropherograms were assembled using Vector NTI v5.1 (Informax™ Invitrogen™ Life Science Software, Frederick, MD, USA).

Determination of HBV genotype

HBV genotypes were determined by the genotyping tool of the National Center for Biotechnology Information (NCBI, US National Library of Medicine, Bethesda, MD, USA, <http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi>), which identifies the genotype based on the viral nucleotide sequences. The genotyping tool works by using BLAST to compare a query sequence to a set of reference sequences for known genotypes.²⁴

Determination of surface and polymerase gene mutations

The genafor/arevir–geno2pheno HBsAg escape/drug resistance tool (Center of Advanced European Studies and Research, Bonn,

Table 1
Demographic data and clinical features of the study patients

Characteristic	Treatment-naïve group	Nucleos(t)ide analog therapy group
Patients, n ^a	185	142
Male, n (%)	120 (64.9)	88 (62.0)
Age, median years (range)	39 (1–76)	42 (13–68)
ALT, median U/l (range)	60.7 (8–874)	76.1 (12–1082)
AST, median U/l (range)	54.7 (13–2510)	58 (13–709)
HBV DNA, median log IU/ml (range)	3.5 log ₁₀ (2.0–6.0)	4.3 log ₁₀ (2.0–>6.0)
HBV genotype (%)	A (0.5); D (99.5)	D (100)
Patient chronic hepatitis B history ^b	Patients with new detection, n = 38 Patients immune tolerant on follow-up, n = 12 Patients within waiting period for 6 months, n = 3 Patients with new initial antiviral treatment, n = 10 Inactive HBV carriers, n = 122 ^c HBeAg-negative CHB patients, n = 132	Patients in the immune tolerant phase, n = 25 Patients in the immune reactive phase, n = 23 HBeAg-negative CHB patients, n = 94
Biopsy status	Patients with Knodell fibrosis scores, n = 23 (score 1 in 15%, 2 in 60%, 3 in 15%, 4 in 5%, and 7 in 5%) Patients without biopsy, n = 162	Patients with Knodell fibrosis scores, n = 61 (score 1 in 17%, 2 in 70%, 3 in 10%, and 5 in 3%) Patients with planned biopsy/waiting for biopsy, n = 32 Patients without biopsy, n = 49 LAM → ADV (32 patients); LAM → ADV → ETV (6 patients); LAM → ADV → ETV → TDF (1 patient); LAM → ADV → TDF (5 patients); LAM → ETV (14 patients); LAM → TDF (1 patient); LAM → LAM + ADV (41 patients); LAM → LAM + ETV (13 patient); ADV → ETV (18 patients); ADV → ETV → TDF (1 patient); ADV → ADV + ETV (9 patients); ETV → ETV + TDF (1 patient) LAM: 27.9 (2–126); LAM + ADV: 25 (8–50); ADV: 16.2 (1–38); ADV + ETV: 18 (8–24); ETV: 13.4 (8–26); ETV + TDF: 6 months (only 1 patient); TDF: 4.2 (3–5)
Therapy status ^d	-	
Treatment duration, median months (range)	-	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; CHB, chronic hepatitis B; LAM, lamivudine; ADV, adefovir; ETV, entecavir; TDF, tenofovir.

^a Serological markers of all patients were found negative for hepatitis C virus and hepatitis D virus.

^b Patient history is recorded as per the European Association for the Study of the Liver clinical practice guidelines.²¹

^c Three patients had undetectable serum HBV DNA levels on quantitative HBV real-time PCR; detection was by in-house HBV PCR.

^d 'Switch' or 'add-on' therapy was decided according to the emergence of drug-resistance (primary or compensatory resistance) or clinical and/or virological breakthrough.

Germany, <http://coreceptor.bioinf.mpi-inf.mpg.de/>) for HBV is a database that has been specifically designed for rapid computer-assisted virtual phenotyping of HBV, and accepts genome (nucleic acid) sequences as input. Geno2pheno searches for homology between input sequences and others already stored in its database, which also stores relevant clinical data for drug resistance and surface gene mutations. The data accumulated by direct sequencing were analyzed either manually or using the geno2pheno tool. The geno2pheno tool searches for HBV drug resistance mutations in the RT domain of the polymerase gene at amino acid positions 80, 169, 173, 180, 181, 184, 194, 202, 204, 215, 233, 236, and 250. However, additional RT amino acid substitutions at positions 84, 85, 214, 237, and 238 were searched for manually.²⁵ The overlapping surface gene segment was searched by the geno2pheno tool, mostly for five amino acid substitutions at positions 137, 141, 144, 145, and 147. Additionally, this region was also searched for mutations at positions 100, 109, 110, and 112–157 manually.⁷ An interpretation of the RT amino acid substitutions was performed first manually and secondly by geno2pheno tool database in the same nucleic acid sequence.

Statistical analysis

The significance of differences between two proportions was determined by Chi-square test with Yates' correction for continuity.

Results

Fifteen typical HBsAg escape mutations were detected in the patients: sY100C, sL109I, sL110V, sS117INST, sP120T, sP127T, sG130R, sS132A, sM133I, sY134N, sC137L, sC137G, sD144E, sG145X, and sG145R (Table 2). The prevalence of typical HBsAg escape mutations found in all CHB patients was 8.3% (27/327). The

frequency of these mutations in the NUC therapy group was 8.5% (12/142) and in the treatment-naïve group was 8.1% (15/185). The difference in frequency of HBsAg escape mutations was not significantly different between the groups; Chi-square with Yates' correction = 0.07; $p > 0.05$ (0.7). In the NUC therapy group, ten different patterns of the typical HBsAg escape mutations were found: sY100C + sL110V, sL109I, sP120T, sP127T, sG130R + sG145X, sS132A + sY134N, sY134N + sG145R, sC137G, sD144E, sG145R; in the treatment-naïve group patients, eight different patterns were found: sL109I, sL110V, sS117INST, sP120T, sP127T, sM133I, sC137L + sG145R, sS143L. Some of the typical HBsAg escape mutations were immune-selected: sP120T, sM133I, sD144E, sS143L, and sG145R, detected in 10 patients. Three of these patients were treatment-naïve and developed LAM + telbivudine (LdT), ADV, and TDF drug resistance; seven patients were in the NUC therapy group and three of these developed LAM, LAM + LdT, and ADV drug resistance, respectively (Table 2). However, the prevalence of the most reported vaccine escape mutation, sG145R, was found to be 1.2% (4/327) in all the CHB patients.

LAM, LdT, ADV, ETV, and TDF drug resistance mutations were detected in the CHB patients: rtL180 M + rtM204I/V, rtM204I, rtA181V/T, rtQ215S, rtI233V, rtN236T, rtL180M + rtS202C + rtM204V, and rtA194T/V. Additionally, in some patients, drug resistance mutations were detected with compensatory mutations: rtL80F, rtL180V, rtA194G, rtV214P, rtQ215H/P, rtI233S, rtN236S, and rtM250R (Table 2). In the treatment-naïve patients with preexisting drug resistance mutations, these were related to LAM (rtL180M + rtM204I), ADV (rtA181V, rtQ215S, rtI233V), ETV (intermediate susceptibility with rtL180M + rtM204I HBV variant), LdT (rtL180M + rtM204I), and TDF (rtA194T) (Table 2). However, NUC resistance mutations were found in 7.7% (11/142) in the NUC therapy group and 3.8% (7/185) in the treatment-naïve group. This distinct

Table 2

Characteristics of HBsAg escape mutation-positive subjects and concomitant nucleos(t)ide analog resistance mutations

Patient	Gender, age (years)	Treatment status	Typical HBsAg escape mutation	Nucleos(t)ide analog resistance mutation	Compensatory mutation	Drug resistance
1	Male, 52	Treatment-naïve	sL109I	rtA181V ^a	-	LAM, ADV
2	Female, 36	Treatment-naïve	sI110V	-	-	-
3	Female, 64	Treatment-naïve	sI110V	-	-	-
4	Female, 54	Treatment-naïve	sI110V	-	rtA194V, rtQ215H	-
5	Female, 44	Treatment-naïve	sS117INST	-	-	-
6	Female, 25	Treatment-naïve	sP120T	rtL180M + rtM204I ^a	-	LAM, LdT
7	Male, 76	Treatment-naïve	sP127T	rtQ215S	-	ADV
8	Female, 52	Treatment-naïve	sP127T	-	-	-
9	Female, 36	Treatment-naïve	sP127T	-	-	-
10	Male, 55	Treatment-naïve	sP127T ^b	rtI233V ^b	-	ADV
11	Male, 48	Treatment-naïve	sP127T	-	-	-
12	Female, 29	Treatment-naïve	sM133I	-	rtI233S, rtM250R	-
13	Female, 62	Treatment-naïve	sL109I	rtM204I	-	LAM, LdT
14	Male, 56	Treatment-naïve	sS143L	rtQ215S	rtA194G	ADV
15	Male, 26	Treatment-naïve	sC137L + sG145R	rtA194T	-	TDF
16	Male, 55	LAM, 36 months	sY100C + sI110V ^b	rtA181T ^{b,c}	-	LAM, ADV
17	Male, 53	LAM, 6 months	sL109I	rtA181V	-	LAM, ADV
18	Male, 35	LAM, 18 months	sP120T	rtL180M + rtM204I ^a	-	LAM, LdT
19	Female, 38	ETV, 3 months	sP127T ^b	rtM204I ^b	-	LAM, LdT
20	Male, 32	LAM, 36 months	sS132A + sY134N	rtL180M + rtS202C + rtM204V	rtV214P	ETV
21	Male, 44	ADV, 48 months	sY134N + sG145R	rtI233V	rtN236S	ADV
22	Female, 32	ADV + LAM, 12 months	-	-	-	-
23	Female, 41	LAM, 36 months	sG130R + sG145X	rtA181V	-	LAM, ADV
24	Female, 41	ADV, 12 months	sC137G	rtL180M + rtM204I, rtI233S	rtL180V	LAM, LdT, ADV
25	Male, 53	LAM, 24 months	sD144E	rtL180M + rtM204V	rtL80F	LAM
26	Male, 14	LAM, 24 months	sD144E	rtQ215S	rtQ215P	ADV
27	Male, 42	ADV, 48 months	sG145R	rtN236T ^c	-	ADV
28	Male, 76	ADV + LAM, 12 months	-	-	-	-
29	Male, 76	LAM, 1 month	sG145R	-	-	-

HBsAg, hepatitis B surface antigen; LAM, lamivudine; ADV, adefovir; LdT, telbivudine; TDF, tenofovir; ETV, entecavir; HBV, hepatitis B virus.

^a According to the European Association for the Study of the Liver clinical practice guidelines 2009, rtA181V-positive and rtL180M + rtM204I-positive HBV variants are intermediately susceptible to LAM and ETV, respectively.^b HBsAg escape and nucleos(t)ide analog resistance mutations detected after in-house HBV PCR in patients 10, 16, and 19.^c Mutations only detected manually: rtA181T in patient 16 and rtN236T in patient 26.

difference between the two frequencies was not significant; Chi-square with Yates' correction = 3.37; $p > 0.05$ (0.066).

The NUC therapy and treatment-naïve group patients had respective values of HBV viral load median 4.3 log₁₀ (2.0–>6.0) and 3.5 log₁₀ (2.0–6.0), alanine aminotransferase (ALT) median 76.1 (12–1082) U/l and 60.7 (8–874) U/l, and aspartate aminotransferase (AST) median 58 (13–709) U/l and 54.7 (13–2510) U/l; these values were not found to be related to HBsAg escape and NUC resistance mutations. However, the medians of these baseline characteristics in the two groups were found to be significantly different according to the Mann–Whitney U-test (HBV viral load median z score = -4.314, $p = 0.000$; ALT median z score = -2.562, $p = 0.010$), with the exception of median AST (z score = -1.470, $p = 0.141$). In addition, patient age and gender were not correlates with the presence of detected mutations.

Knodell's fibrosis scores of the NUC therapy and treatment-naïve group patients were not correlated between the two groups or with the presence of detected mutations.

Direct sequencing results revealed HBV genotype D in all patients, except for one patient with genotype A – an inactive HBV carrier in the treatment-naïve group (Table 2).

Discussion

The typical HBsAg escape mutations detected in this study (sP120T, sG130R, sM133I, sY134N, sD144E, sS143L and sG145R) have been shown in many investigations to cause diagnostic problems in HBsAg assays and vaccine or HBV therapy escape.^{5,7,26,27} However, insufficient data are available for interpretation, and the clinical effects of some of the detected typical HBsAg escape mutations found in this study (i.e., sY100C, sL109I, sI110V, sS117INST, sP127T, sS132A, sC137L, sC137G, sG145X) are

not clear in the background literature.⁷ Fifteen typical HBsAg escape mutations were detected in our study patients, and these were single or multiple. To date, Turkish data have been limited to case studies and this is the first report of HBsAg escape mutations in Turkish CHB patients. In most geographical regions, the prevalence of these mutants in the population of HBV carriers is unknown. By DNA sequence analysis, a frequency close to 33% has been found in Korean carriers, 38% in Gambian carriers, and 40% in Spanish carriers. sP120T, sL127P, sG129H, sM133I/T, sP/T134A/L, sS140L, and sG145A/R were the most frequent HBsAg escape mutations representing the 'a' determinant of the polymerase protein of HBV in these studies.^{7,28} The frequency of typical HBsAg escape mutations according to polymerase gene sequencing of HBV found in our study is lower than that found in other studies.^{7,28} This higher detected frequency in the other studies could be as a result of using the DNA sequencing method to focus on all amino acid substitutions in the surface gene of HBV. On the other hand, clonal analysis may have been used in these studies and this approach can provide more information, especially whether the mutation is on the same genome. Recently, the first vaccine escape mutation (sT143*) was identified in Turkey, concomitantly with other HBsAg escape mutations (sG145A, sP142S), in a child with CHB who had been vaccinated against HBV.¹⁹ The study of Sayiner et al. showed five amino acid mutations (sS143L, sQ101H, sS117N, sT118R, sP120Q) in a treatment-naïve and previously vaccinated Turkish patient.²⁰ However, in our previous study, we demonstrated naturally occurring RT sequence changes related to NUCs in treatment-naïve Turkish patients with CHB.²⁹ There has been no information in Turkey on the prevalence and pattern of typical HBsAg escape mutations and concomitant NUC resistance mutations in different antiviral therapies and in treatment-naïve patients with CHB.

The emergence of natural mutations should be expected due to the characteristics of the HBV genome. The major causes of drug resistance include viral factors, such as the kinetics of viral production and clearance, and the lack of a proofreading mechanism during reverse transcription, which creates a large HBV quasi-species pool and replication fitness of the viral quasi-species.³⁰ The prevalence of typical HBsAg escape mutations in our treatment-naïve patients was 8.1% and the patterns were sL109I, sI110V, sS117INST, sP120T, sP127T, sM133I, sC137L + sG145R, and sS143L. Interestingly, preexisting drug resistance mutations were related to LAM (rtL180M + rtM204I, intermediate susceptibility with rtA181 V HBV variant), ADV (rtA181V, rtQ215S, rtI233V), ETV (intermediate susceptibility with rtL180M + rtM204I HBV variant), TDF (rtA194T), and LdT (rtL180M + rtM204I) in a few patients who were candidates for treatment of their CHB according to current EASL guidelines.²¹ These data suggest that the circulation of HBV encoding surface mutations with concomitant NUC-associated resistance variants is possible in Turkey. This is particularly the case for the populations with the highest prevalence rates, in southeastern Turkey. However, evidence for the spread or transmission of NUC-resistant HBV is limited.⁹ In addition, HBV encoding LAM-resistant mutations has also been found in a cohort of dialysis patients with occult HBV.³¹ On the other hand, Ozaslan et al. reported a high secondary mutation rate for the HBV surface gene in family members of HBV patients in Turkey.¹⁵ Therefore, monitoring is important in order to determine whether or not variants can be transmitted and whether they represent an important public health threat.

The concern is that vaccine escape variants may enhance resistance to NUCs such as LAM and ADV, and that NUC-resistant viruses may also, coincidentally, be vaccine escape variants.³² A triple mutation pattern related to LAM resistance (rtV173L + rtL180 M + rtM204 V) has been shown to cause two amino acid changes in the overlapping surface gene (sE164D + sI195M), which reduce anti-HBs binding to levels seen only with the vaccine escape mutant sG145R.^{33,34} Selection of an sP120A mutation in these patients is associated with apparent HBsAg seroconversion. This mutation produces a reduced anti-HBs binding, which explains the failure to detect HBsAg.^{5,35} Some of the typical HBsAg escape mutations have been described as immune-selected mutations: sI20T, sM133I, sS143L, sD144E, and sG145R.⁵ In this study, these were detected in 10 patients and were predominant in the NUC therapy group. According to our knowledge, the sS143L mutation was the third mutation identified in Turkey, and was detected by HBsAg assays used in routine laboratories. However, all detected immune-selected mutations appeared alone. Some of these patients developed LAM, LAM + LdT, and ADV drug resistance (Table 2). According to recently published studies, envelope changes can be detected with LAM, ADV, ETV, and TDF resistance.^{9,36} Vaccine/HBIg escape mutations sP120T and sG145R in combination with drug resistance mutations related to LAM are often seen in HBV-mono-infected patients following LAM or HBIg treatment.⁸ They produce changes, rtT128N and rtW153Q, respectively, in the polymerase gene and have been found to partially restore the *in vitro* replicative capacity of LAM-resistant HBV.^{5,33} At the same time, the sM133I immune-selected mutation induces changes in the rtV142I mutation in the overlapping polymerase gene. However, there are insufficient data available for interpretation of the mutation rtV142I. The choice of first treatment strategy may affect the efficacy of future treatment options.³⁷ Therefore, NUC resistance mutations should not be selected by HBV encoding envelope mutations, particularly surface mutations, and vice versa, for the effective treatment of CHB.

In this study the rtA194T drug resistance mutation was detected as a preexisting mutation, and the rtN236T drug

resistance mutation was detected in those under NUC therapy. Neither the ADV-associated resistance mutation rtN236T nor the TDF-associated resistance mutation rtA194T causes changes in the HBV surface gene.^{9,38,39} Interestingly, the sG145R escape mutation was detected in both groups of patients (Table 2). Therefore, it is possible to say that the sG145R escape mutation occurred naturally in these patients. According to our results, the sG145R escape mutation prevalence was detected at a low frequency (1.2%) in Turkish patients with CHB. Amino acid changes in residue 145 have been found at a low frequency (0.4%, 1/272) in HBV carriers in Spain.⁷ Naturally occurring mutants of the residue 145 are difficult to obtain, but are required in HBsAg seroconversion mutant panels. Manufacturers of commercially available seroconversion panels are enhancing the ability of the assays to detect HBs mutants in sufficient amounts to allow for comparisons with different HBsAg assays on the market.^{26,40} The variant sG145R was identified initially as a vaccine escape mutant.²⁷ It has been demonstrated that it is also associated with HBV-related chronic liver disease in non-vaccinated subjects.^{27,41} On the other hand, the horizontal transmission of the sG145R mutant strain has been reported.⁴² The patients in this study with sG145R mutant strains had never been vaccinated and had not received HBIg prior to the development of their HBV infection. However, naturally occurring surface gene variants have also been reported around the world in persons who have not been immunized.^{43,44}

The replication defects in HBV can partially be compensated by selection according to secondary (compensatory) mutations.⁴ Some of these compensatory mutations (rtL80F, rtV214P, rtQ215H/P, rtI233S, rtN236S) were detected in this study, usually in patients under LAM and/or ADV therapy, and most of these are associated with ADV failure.²⁵ According to our previous study, such compensatory mutations can occur in treatment-naïve patients.²⁹ Furthermore, variations at rtQ215 have been detected relatively frequently, affecting treatment-naïve and LAM-pre-treated patients.⁴⁵ In particular, the rtQ215H substitution has also been observed in patients receiving LAM or ADV therapy, but its virological and clinical relevance remain unclear.⁴⁶ In this study, no close relationship between compensatory mutations and HBV viral loads was found. Viral load may be an indirect marker of replication efficiency. Nevertheless, detailed evaluation of the replication capacity was not one of the aims of this study.

In a recent study, it was demonstrated that mutations in the HBsAg selected by LAM therapy developed more frequently in HBV genotype A when compared with genotype D.³⁶ Several studies have shown genotype D of HBV to represent almost the whole Turkish patient population infected with HBV.^{14,17,29,47} The present study showed that genotype D of HBV is still dominant among Turkish HBV-infected patients; genotype A was found in only one patient who was an inactive HBV carrier. As our group was genotype D dominant, it was not possible to make a comparison of mutations related to the different HBV genotypes. However, it would be worthwhile to see if mutations related to HBsAg escape occur more frequently in HBV infections of some genotypes than others.

In this study, an interpretation approach, manual analysis, and an HBsAg escape/drug resistance tool were used. The results of the manual and geno2pheno tool analyses were similar except in the cases of two patients with ADV drug resistance (rtA181T and rtN236T, in patients 16 and 26, respectively) (Table 2). In the DNA chromatogram of these patients, two peaks were obtained (wild and mutant type) for the same nucleotide signal (data not shown). Due to the algorithm used in the HBsAg escape/drug resistance tool, the most prominent signal is defined as a nucleotide. Therefore, one must be careful when using such a tool in order to avoid inaccuracies. However, manual analysis and the HBsAg escape/drug resistance tool can be used together for interpretation.

On the other hand, the geno2pheno is a useful tool that enables a simple and rapid analysis for the identification of mutations associated with HBsAg escape and resistance to NUCs in large regions of the HBV genome sequence, as was done in this study.

In this study we focused on the typical HBsAg escape mutations via DNA sequencing of the polymerase gene region. By this method it is possible to monitor NUC resistance mutations concomitantly in the same sequencing. In conclusion, our findings show preexisting typical HBsAg escape and NUC resistance mutations are possible. Drug resistance mutations in the HBV polymerase gene may not always result in a direct impact on the nature of HBsAg and its function, and vice versa. However, the genetic arrangement of the HBV genome with polymerase and surface genes overlapping has substantial public health and diagnostic implications and relevance.

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